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Distribution and hybridisation of barred and common grass snakes (*Natrix helvetica*, *N. natrix*) in Baden-Württemberg, South-western Germany

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Abstract

The distribution and hybridisation zone of the two grass snake species occurring in the German state of Baden-Württemberg are described, based on genetic data from maternally inherited mitochondrial DNA (mtDNA, up to 1983 bp) and biparentally inherited microsatellite DNA (13 loci). In agreement with previously published morphological evidence, the barred grass snake (*Natrix helvetica*) occurs in the Upper Rhine Valley and the Black Forest, while the common grass snake (*N. natrix*, 'yellow lineage') is distributed across the remaining, more eastern parts of Baden-Württemberg. Cline analyses across two transects running through the region of Karlsruhe and the Black Forest indicate that the hybrid zone is similarly narrow here as in the previously characterised stretch near Lake Constance. With respect to nuclear DNA, the Black Forest constitutes no impediment to gene flow in comparison with lowland regions (Karlsruhe, Lake Constance). However, on the eastern slope of the Black Forest, the abrupt replacement of mtDNA of *N. helvetica* by that of *N. natrix* indicates male-mediated gene flow and that the Black Forest represents a dispersal barrier for female grass snakes.

Key Words

cline analysis, gene flow, hybrid zone, Natricidae, Serpentes

Introduction

Grass snakes were, until recently, thought to represent a widespread species distributed from North-western Africa and the Iberian Peninsula across much of Western, Central, Northern and Eastern Europe into Central Asia (Kabisch 1999). Genetic investigations revealed three deeply divergent genealogical lineages without or with very restricted, unidirectional gene flow and prompted the recognition of three distinct species (Kindler et al. 2013, 2014, 2017, 2018a, b; Pokrant et al. 2016). According to these investigations, the red-eyed grass snake, *Natrix astreptophora* (Seoane, 1884), occurs in North-western Africa, the Iberian Peninsula and South-western France. Its range abuts in the French pre-Pyrenean region that of the barred grass snake *Natrix helvetica* (Lacepède, 1789). Hybridisation is very rare, and the distribution of the two species seems to be mutually exclusive (Pokrant et al. 2016). *Natrix helvetica* is a polytypic species (Kindler and Fritz 2018) distributed over France, the Benelux countries, Great Britain, Switzerland, Western Germany, and Italy. The barred grass snake hybridises in a narrow belt, largely corresponding to the Rhine region, with the common grass snake, *Natrix natrix* (Linnaeus, 1758), whose intraspecific systematics is poorly understood. There are many conflicts between traditionally recognised subspe-

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cies and genetic lineages and clusters (Kindler et al. 2013, 2017). Gene flow is mainly unidirectional from *N. helvetica* into *N. natrix*. The common grass snake occurs from Central Europe through the Balkans, the Near and Middle East to Central Asia (Kindler et al. 2017).

While this general picture is now well established, many regions were undersampled in the aforementioned studies, leaving open many questions about the exact distribution of the three grass snake species and the location of contact zones. It has long been known that barred and common grass snakes occur in the German state of Baden-Württemberg (Waitzmann and Sowig 2007; Laufer 2008). This state lies in the southwest of Germany and has an area of approximately 36,000 km^2. Baden-Württemberg abuts Switzerland in the south and France in the west. To the north and east, Baden-Württemberg shares borders with the German states of Rhineland-Palatinate, Hesse, and Bavaria. In the west of Baden-Württemberg, the Black Forest mountains, reaching an elevation of approximately 1500 m, separate the Upper Rhine Valley from the eastern parts of the state, which are divided by the southwest-northeast trending Swabian Jura, with the highest peaks reaching approximately 1000 m. South of the Swabian Jura, rivers drain generally into the Danube system, while north of the mountain chain and in the Upper Rhine Valley, rivers belong to the catchment basin of the Rhine (Gebhardt 2007).

The barred grass snake is distributed in Baden-Württemberg in the Upper Rhine region and in the Black Forest, while the common grass snake occurs in the eastern parts of the state (Waitzmann and Sowig 2007; Laufer 2008). However, neither the exact distribution of the two species nor the location of their putative hybrid zone in the west of Baden-Württemberg has been examined genetically. Based on morphological evidence, it has been suggested that the hybrid zone runs roughly from the region of Neckargemünd across the eastern foothills of the Black Forest to Waldshut-Tiengen (Laufer 2008). In the present study, we clarify for Baden-Württemberg the exact distribution of the two grass snake species and locate their contact zone. In order to do so, we follow the approach of Kindler et al. (2017) with respect to the chosen genetic markers and use maternally inherited mitochondrial DNA (mtDNA) sequences of up to 1983 bp length in combination with information derived from length polymorphisms of 13 biparentally inherited microsatellite loci. In addition, we examine the extent of gene flow between the two taxa using cline analyses across two transects based on both marker systems.

Material and methods

A total of 93 grass snake samples from Baden-Württemberg was studied (Suppl. material 1: Table S1). Data for 50 of these samples were available from Kindler et al. (2017), while the remaining material was processed for the present study. The new samples originate from road-kills or shed skins collected by volunteers for this study. The chosen genetic markers were the same as in Kindler et al. (2017). With respect to mtDNA, the partial ND4 gene plus adjacent DNA coding for tRNAs (ND4+tRNAs, up to 866 bp) and the cytochrome *b* gene (cyt *b*, up to 1117 bp) were sequenced. As nuclear genomic markers, 13 informative polymorphic microsatellite loci were used to genotype the same samples. Laboratory approaches were described in detail in Kindler et al. (2013) and Pokrant et al. (2016). For embedding the genetic variation within Baden-Württemberg into a broader framework, data for additional 421 grass snakes from adjacent regions in the Czech Republic, France, Germany, and Switzerland were included in all calculations (Suppl. material 1: Table S1). Except for seven newly processed samples, these data were from Kindler et al. (2017).

Mitochondrial DNA sequences were assigned to one of the clades identified by Kindler et al. (2013, 2017) by running exploratory Maximum Likelihood trees using MEGA 7 (Kumar et al. 2016) and the same outgroups as in Kindler et al. (2018a). In addition, haplotype networks were built using POPART (http://popart.otago.ac.nz) and the implemented parsimony network algorithm of TCS (Clement et al. 2000). For the microsatellite data, unsupervised analyses were run using STRUCTURE 2.3.2.1 (Falush et al. 2007; Hubisz et al. 2009) assuming the admixture model and correlated allele frequencies. This approach uses only genetic evidence for inferring genetic structuring, without any *a priori* information (collection site, geographic distances) and allows the identification of individual admixed or pure genotypes within population clusters. All calculations were run for K = 1–10 and repeated 10 times for each K, using a MCMC chain of 1,000,000 generations for each run and a burn-in of 250,000 generations. The most likely number of clusters (K) was determined by the ΔK method (Evanno et al. 2005) using the software STRUCTURE HARVESTER (Earl and vonHoldt 2012). Population structuring and individual admixture were visualised in DISTRUCT 1.1 (Rosenberg 2004). The same model-based threshold for admixed individuals (5%) as in Kindler et al. (2017) was used for further processing.

The STRUCTURE results and the mitochondrial identity of the samples were then used for running cline analyses in HZAR (Derryberry et al. 2014) as described in Kindler et al. (2017). Cline analyses measure the transition of distinct traits, in our case genetic traits, across a contact zone of distinct taxa and facilitate the understanding of genetic introgression, hybridisation and the transition from one character state (or genetic identity) to another. In taxa with high dispersal capability, like grass snakes (Madsen 1984; Meister et al. 2012; Wisler et al. 2008), steep and narrow clines indicate incipient or complete reproductive isolation, while shallow and wide clines are typical for broad-scale gene flow (Barton 1979; Derryberry et al. 2014; Rieseberg et al. 1999).

For the cline analyses, two transects were selected, one running across the Black Forest and the other north of the

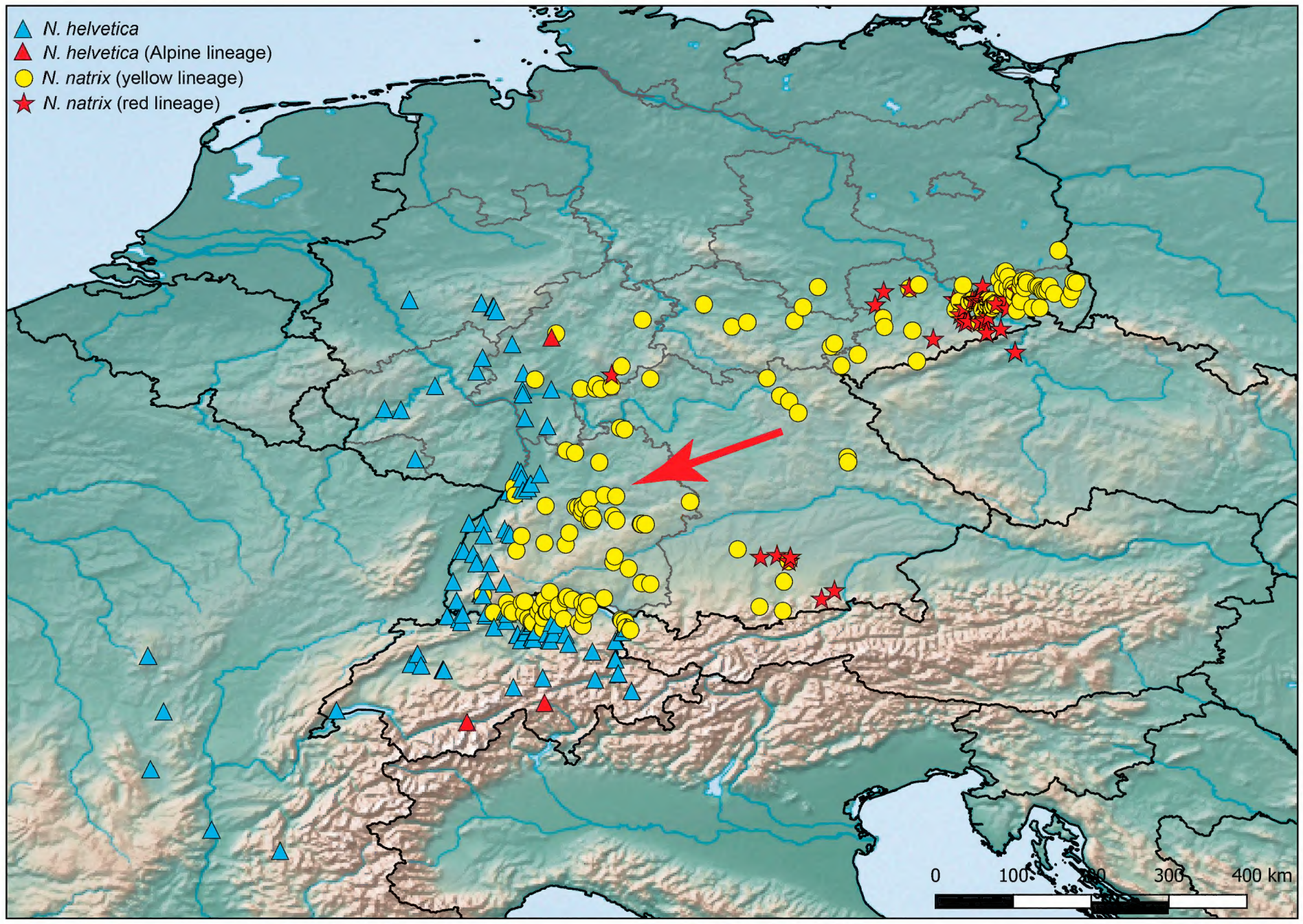

Figure 1. Distribution of mitochondrial lineages of *Natrix helvetica* and *N. natrix* in the study area. The red arrow highlights Baden-Württemberg. The record of the Alpine lineage of *N. helvetica* in Hesse refers to an introduced grass snake (Kindler et al. 2017).

Black Forest through the lowland region of Karlsruhe that connects the Upper Rhine Valley with the more eastern regions. Using QGIS (http://qgis.org), samples from within 50 km left and right of the transect were arbitrarily lumped together, if material from more than one locality within this stretch was available. For microsatellites, mean proportions of cluster membership (Q values) were used for each site. For mitochondrial data, the frequency of haplotypes of *N. helvetica* or *N. natrix* ('yellow' and 'red lineages' lumped together) was used. For both datasets (mtDNA, microsatellites), clines were calculated for each transect. The settings and further processing of the HZAR calculations were the same as in Kindler et al. (2017).

Results

Based on the exploratory Maximum Likelihood trees and networks (not shown), the mitochondrial haplotypes of all samples from Baden-Württemberg could be unambiguously assigned either to *Natrix helvetica* or to the 'yellow lineage' (Kindler et al. 2017) of *N. natrix* (Suppl. material 1: Table S1). Haplotypes of the 'red lineage' of *N. natrix* (Kindler et al. 2017) have not been recorded from Baden-Württemberg but from Bavaria and Hesse (Kindler et al. 2017).

Besides known haplotypes (Suppl. material 1: Table S1), for *N. helvetica* two new haplotypes for ND4+tRNAs were identified in Baden-Württemberg (h13, h14; European Nucleotide Archive accession numbers LR694446, LR694447). For *N. natrix*, three new haplotypes of the 'yellow lineage' were found (y44-y46; LR694443-LR694445). For the cyt *b* gene, there were two new haplotypes for *N. helvetica* (h30-h31; LR694440, LR694441) and one for the 'yellow lineage' of *N. natrix* (y43; LR694442). Each of the new haplotypes was a tip haplotype, differing by one mutation step from a common central haplotype (h1 and y1, respectively) in networks (see fig. 2 in Kindler et al. 2017).

In Baden-Württemberg, the haplotypes of the two species are largely parapatrically distributed, with haplotypes of *N. helvetica* confined to the westernmost parts of the state (Upper Rhine Valley, Black Forest) and haplotypes of *N. natrix* distributed over the remaining central and eastern regions, including the Lake Constance area (Fig. 1). Along the south-easternmost slopes of the Black Forest and in the region of Karlsruhe, the distribution of the haplotypes of the two species overlaps.

As expected, STRUCTURE HARVESTER revealed $K = 2$ as the most likely number of genomic clusters for the processed dataset. One cluster corresponded to barred grass snakes and the other to common grass snakes. Based

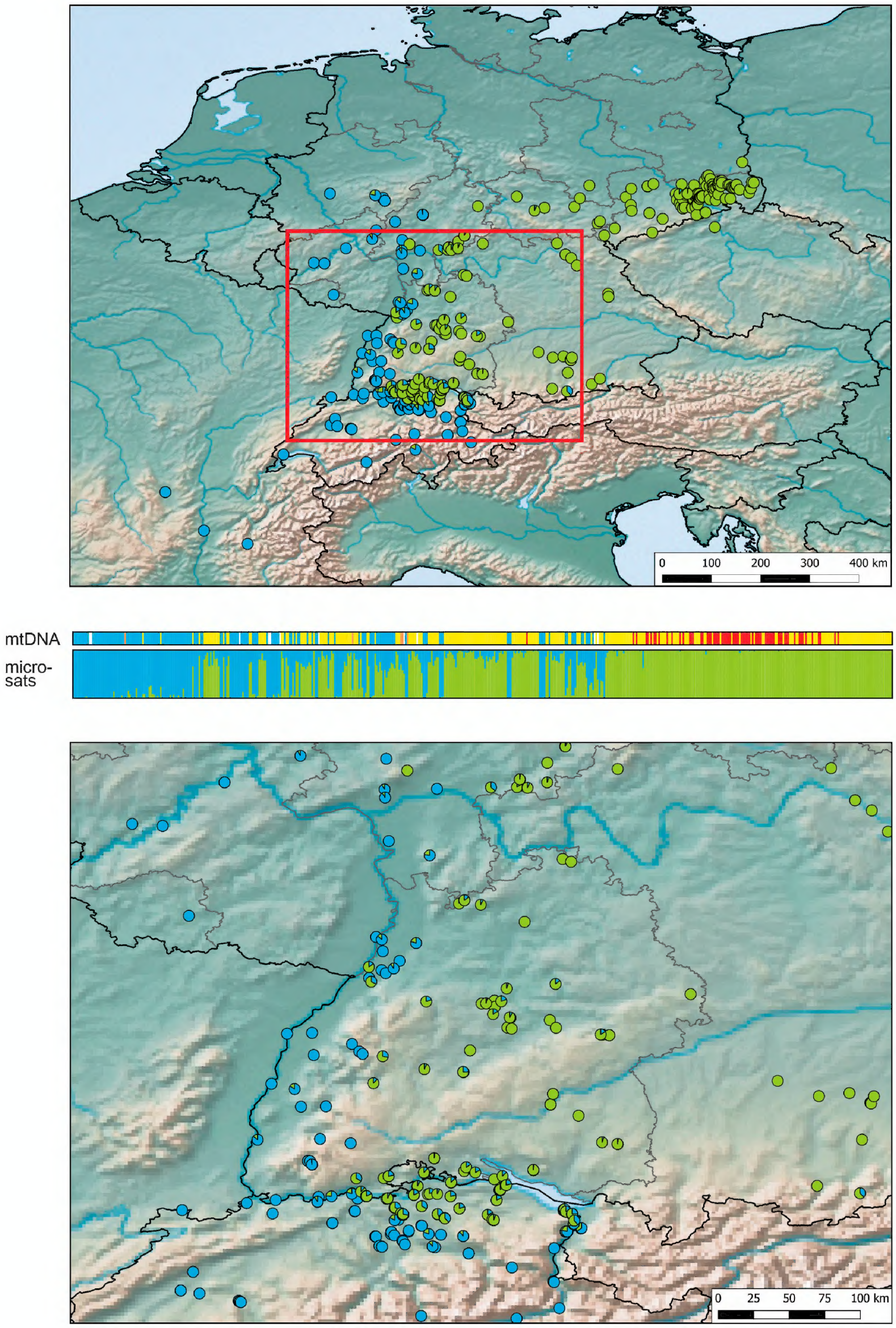

Figure 2. Genotypic identity of grass snakes in the study area. Top: Whole sampling used; blue corresponds to *Natrix helvetica*; green, *N. natrix*. The red rectangle highlights the enlarged map segment shown below. Centre: Mitochondrial identity (upper bar) and genotypes (lower bar) of individual grass snakes. Samples are arranged from west to east. The upper bar indicates the mitochondrial haplotype of each individual. Colours represent *N. helvetica* (blue), the 'Alpine lineage' of *N. helvetica* (orange), the 'yellow lineage' of *N. natrix* (yellow) and the 'red lineage' of *N. natrix* (red). White segments represent missing mtDNA data. STRUCTURE results are shown in the lower bar. There, each vertical column corresponds to an individual snake; colours indicate its genotypic identity for $K = 2$ (blue = *N. helvetica*, green = *N. natrix*). Divided columns with percentages > 5% represent snakes with admixed ancestry. Note that there are many individuals with haplotypes of *N. natrix* with genomic introgression from *N. helvetica,* but few snakes harbouring haplotypes of *N. helvetica* with genomic introgression from *N. natrix*. Bottom: Detailed map for Baden-Württemberg and adjacent regions.

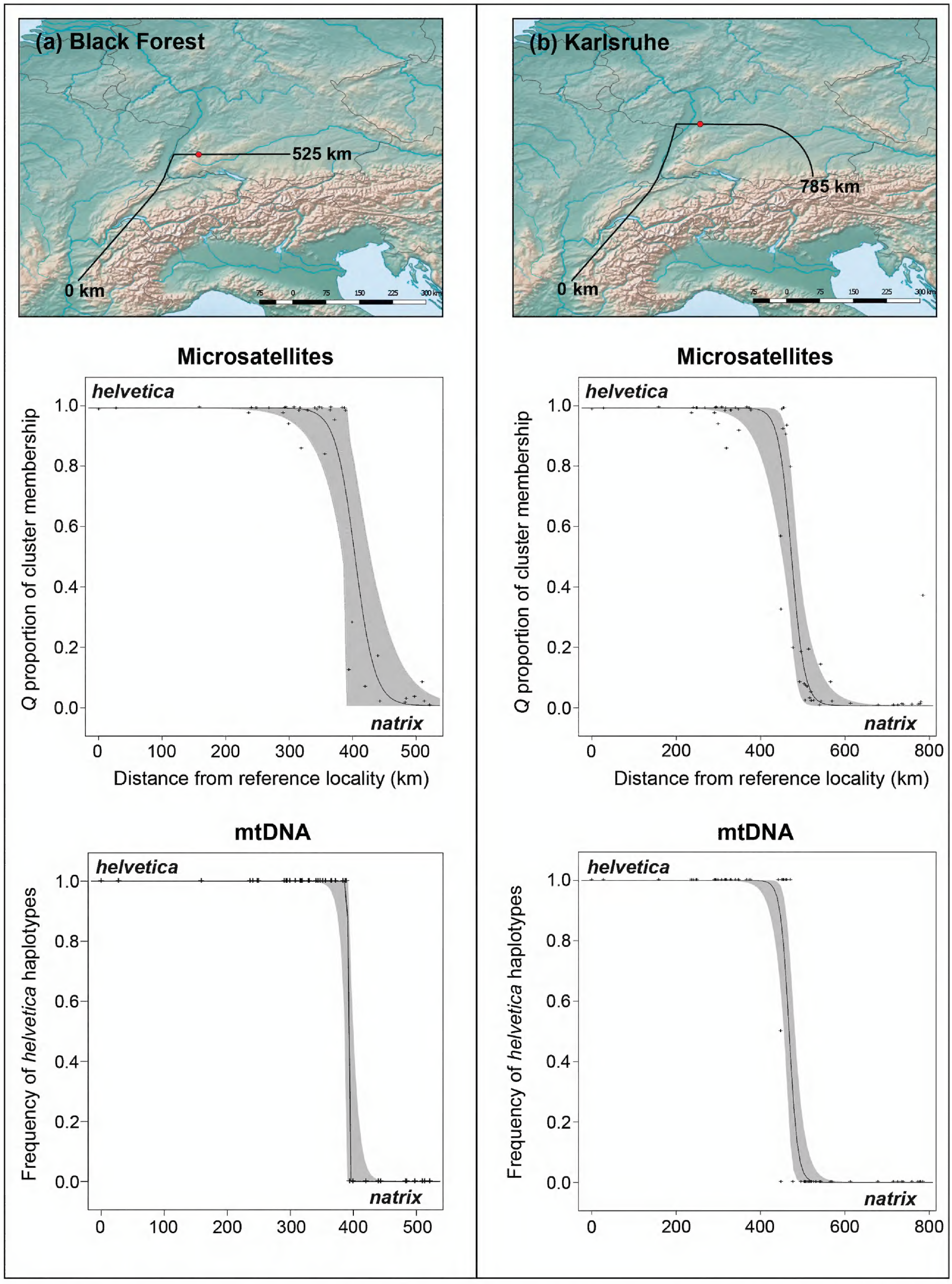

Figure 3. Cline analyses for microsatellites and mtDNA sequences across the two transects running through Baden-Württemberg (top). The red dots in the maps represent the cline centres. The Maximum Likelihood clines are shown for *Q* values of cluster membership (microsatellites, centre) and the mitochondrial identity (bottom) of samples. Grey indicates the fuzzy cline regions (95% credibilities). Note the steep and concordant clines for both marker systems.

on STRUCTURE analyses of our whole dataset, it is obvious that hybridisation between the two species is geographically largely restricted to a narrow strip following roughly the upper course of the Rhine and deviating to the southeast within Switzerland (Fig. 2, top). To the north, the hybrid zone is located east of the Rhine, and along the Main River and other eastern tributaries of the Rhine, the genetic impact of *N. helvetica* reaches further eastwards. An isolated record in Southern Bavaria results from hybridisation between *N. natrix* and the 'Alpine lineage' of *N. helvetica* (Kindler et al. 2017; Glaw et al. 2019). Within Baden-Württemberg, admixture between the two species is largely restricted to three narrow regions: (1) the area around Karlsruhe, (2) the eastern slope of the Black Forest, and (3) the Lake Constance region (Fig. 2, bottom). The Lake Constance region was examined in detail by Kindler et al. (2017). These authors concluded that hybridisation is restricted to a narrow, approximately 50-km-wide, zone that lies mainly within Switzerland. In the following, we present cline analyses for the other two regions.

The chosen transect across the Black Forest is 525 km long and extends in southwest-northeast direction from Southern France to the Upper Rhine Valley across the Black Forest to Bavaria. The transect running through the Karlsruhe region is 785 km long and extends in the Upper Rhine Valley further north and extends further to the southeast. The clines for mtDNA and STRUCTURE cluster membership in both contact zones are highly concordant and very steep (Fig. 2), indicating an abrupt genetic transition.

For the Black Forest, the cline centre for microsatellites lies 403.8 km from the reference locality used as the starting point (95% confidence interval 381.5–432.0 km) with a cline width of 61.9 km (95% confidence interval 0.1–145.9 km). The cline centre for mtDNA is 391.1 km distant from the reference point (95% confidence interval 387.6–397.4 km) and the cline width is only 0.2 km (95% confidence interval 0–25.1 km). For the cline running across the Karlsruhe region, the centre for microsatellites is 473.2 km from the reference locality (95% confidence interval 449.9–489.7 km) and the cline width is 60.9 km (95% confidence interval 30.0–149.9 km). For mtDNA, the cline centre is 467.7 km (95% confidence interval 454.6–482.1 km) from the reference locality; the cline width is 40.3 km (95% confidence interval 19.5–81.9 km).

Discussion

Our study genetically confirms the occurrence of two species of grass snake in Baden-Württemberg and defines, for the first time, the exact location of the contact zone of *Natrix helvetica* and *N. natrix*, in which the two species hybridise. The contact zone runs from the region of Karlsruhe along the eastern slope of the Black Forest to the Lake Constance region. In the Lake Constance region, the narrow hybrid zone lies mostly in Switzerland (Kindler et al. 2017) and only its northernmost extension encroaches into Baden-Württemberg. This is also reflected by our finding that only mitochondrial haplotypes of *N. natrix* (yellow lineage) are present in this region of southernmost Baden-Württemberg.

According to our results, the hybrid zone near Karlsruhe has a width of approximately 40–60 km (mtDNA, microsatellites) and on the eastern slope of the Black Forest, approximately 0–60 km (mtDNA, microsatellites). This resembles the width of the hybrid zone in Switzerland (approx. 40 km both for mtDNA and microsatellites; Kindler et al. 2017). The similarity of the three transects shows that the hybrid zone is generally narrow, about 50 km wide, and that the Black Forest represents no pronounced barrier against nuclear gene flow. If the Black Forest constituted a barrier, a narrower hybrid zone would be expected there. On the other hand, this pattern also implies that intrinsic barriers against hybridisation override the impact of topography, resulting in similar patterns in lowland regions without mountain chains (Karlsruhe, Lake Constance) and around the Black Forest. However, on the eastern slope of the Black Forest, the abrupt replacement of mtDNA of *N. helvetica* by that of *N. natrix* suggests that gene flow is completely male-mediated and that the Black Forest effectively prevents the dispersal of female grass snakes.

Our results are in line with the general picture drawn by Kindler et al. (2017). In the narrow hybrid zone of *N. helvetica* and *N. natrix*, pure or little admixed parental genotypes occur in close geographic proximity, together with hybrids. This corresponds to a bimodal hybrid zone (Jiggins and Mallet 2000) in which gene flow is mainly unidirectional from *N. helvetica* into *N. natrix* and thus male-mediated (Kindler et al. 2017; this study). The whole situation indicates that the speciation process of the two taxa is widely advanced and has resulted in largely discrete gene pools.

A challenge for nature conservation will be to maintain the natural distribution pattern of the two species with their narrow hybrid belt. Grass snakes are known to be highly mobile and disperse easily, even across intensively used terrestrial habitat (Madsen 1984; Meister et al. 2012; Wisler et al. 2008), so that it can be concluded that the hybrid zone is stable. Therefore, an imminent risk is posed by the translocation of grass snakes across the hybrid zone, for instance during compensation measures for road constructions or when rescued snakes, removed from properties in settlements, are released. Even more serious is to release grass snakes of unknown provenance, e.g. confiscated terrarium animals, because this can lead to 'genetic pollution' of native populations with alien genetic material. Considering the high dispersal ability of grass snakes (Meister et al. 2012) with average home ranges of 25–40 ha (Madsen 1984; Wisler et al. 2008), this threat should not be underestimated. In Britain, Germany, and Switzerland, several cases of introduced grass snakes belonging to foreign genetic lineages are already known, and there is evidence for reproduction and hybridisation with native grass snakes (Dubey et al. 2017; Kindler et al. 2017), resulting in the local loss of the pure native lineages.

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Supplementary material 1

Table S1

Authors: Nadine Schultze, Hubert Laufer, Carolin Kindler, Uwe Fritz

Data type: Table.

Explanation note: Grass snake samples used for the present study.

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